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(54) Title: SYNTHETIC LEADER PEPTIDE SEQUENCES

(57) Abstract

The present invention relates to synthetic leader peptide sequences wherein N–linked glycasylation is lacking. The leader peptide (LP) is used in a DNA construct encoding a polypeptide having the structure: SP–LP–(DS)–(S)–(PS)–*gene*. The construct may be used in yeast.

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SYNTHETIC LEADER PEPTIDE SEQUENCES

FIELD OF INVENTION

The present invention relates to novel synthetic leader peptide sequences for secreting polypeptides in yeast.

BACKGROUND OF THE INVENTION

Yeast organisms produce a number of proteins which are synthesized intracellularly, but which have a function outside the cell. Such extracellular proteins are referred to as secreted proteins. These secreted proteins are expressed initially inside the cell in a precursor or a pre-protein form containing a pre-peptide sequence ensuring effective direction of the expressed product (into the secretory pathway of the cell) across the membrane of the endoplasmic reticulum (ER). The pre-sequence, normally named a signal peptide, is generally cleaved off from the desired product during translocation. Once entered in the secretory pathway, the protein is transported to the Golgi apparatus. From the Golgi the protein can follow different routes that lead to compartments such as the cell vacuole or the cell membrane, or it can be routed out of the cell to be secreted to the external medium (Pfeffer, S.R. and Rothman, J.E. Ann.Rev.Biochem. 56 (1987) 829-852).

Several approaches have been suggested for the expression and secretion in yeast of proteins heterologous to yeast. European published patent application No. 88 632 describes a process by which proteins heterologous to yeast are expressed, processed and secreted by transforming a yeast organism with an expression vehicle harbouring DNA encoding the desired protein and a signal peptide, preparing a culture of the transformed organism, growing the culture and recovering the protein from the culture medium. The signal peptide may be the signal peptide of the desired protein

itself, a heterologous signal peptide or a hybrid of native and heterologous signal peptides.

A problem encountered with the use of signal peptides heterologous to yeast might be that the heterologous signal peptide does not ensure efficient translocation and/or cleavage ot the precursor polypeptide after the signal peptide.

The Saccharomyces cerevisiae MFα1 (α-factor) is synthesized as a pre-pro form of 165 amino acids comprising signal- or pre-peptide of 19 amino acids followed by a "leader" or pro-peptide of 64 amino acids, encompassing three N-linked glycosylation sites followed by (LysArg((Asp/Glu)Ala)$_{2-3}$α-factor)$_4$ (Kurjan, J. and Herskowitz, I. Cell 30 (1982) 933-943). The signal-leader part of the pre-pro MFα1 has been widely employed to obtain synthesis and secretion of heterologous proteins in S. cerevisiae.

Use of signal/leader peptides homologous to yeast is known from i.a. US patent specification No. 4,546,082, European published patent applications Nos. 116 201, 123 294, 123 544, 163 529 and 123 289 and DK patent application No. 3614/83.

In EP 123 289 utilization of the S. cerevisiae α-factor precursor is described whereas WO 84/01153 indicates utilization of the S. cerevisiae invertase signal peptide and DK 3614/83 utilization of the S. cerevisiae PH05 signal peptide for secretion of foreign proteins.

US patent specification No. 4,546,082, EP 16 201, 123 294, 123 544 and 163 529 describe processes by which the α-factor signal-leader from S. cerevisiae (MFa1 or MFα2) is utilized in the secretion process of expressed heterologous proteins in yeast. By fusing a DNA sequence encoding the S. cerevisiae MFα1 signal/leader sequence at the 5' end of the gene for the desired protein secretion and processing of the desired protein was demonstrated.

EP 206 783 discloses a system for the secretion of polypeptides from S. cerevisiae using an α-factor leader sequence which has been truncated to eliminate the four α-factor units present on the native leader sequence so as to leave the leader peptide itself fused to a heterologous polypeptide via the α-factor processing site LysArgGluAlaGluAla. This construction is indicated to lead to an efficient processing of smaller peptides (less than 50 amino acids). For the secretion and processing of larger polypeptides, the native α-factor leader sequence has been truncated to leave one or two of the α-factor units between the leader peptide and the polypeptide.

A number of secreted proteins are routed so as to be exposed to a proteolytic processing system which can cleave the peptide bond at the carboxy end of two consecutive basic amino acids. This enzymatic activity is in S. cerevisiae encoded by the KEX 2 gene (Julius, D.A. et al., Cell 37 (1984b) 1075). Processing of the product by the KEX 2 protease is needed for the secretion of active S. cerevisiae mating factor α1 (MFα1 or α-factor) whereas KEX 2 is not involved in the secretion of active S. cerevisiae mating factor a.

Secretion and correct processing of a polypeptide intended to be secreted is obtained in some cases when culturing a yeast organism which is transformed with a vector constructed as indicated in the references given above. In many cases, however, the level of secretion is very low or there is no secretion, or the proteolytic processing may be incorrect or incomplete resulting in secretion of a considerable amount of leader bound product polypeptide. Prosequences, and especially N-terminally located prosequences, or leader sequences expressed in eucaryotic cells, such as yeast cells, are extensively glycosylated, cf. Fiedler and Simons, Cell, 81, p 309-312; and Moir, D.T., Yeast mutants with increased secretion efficiency, in Yeast Genetic Engineering, Barr, P. J., Brake, A. J., and Valenzuela, P. eds., wherein a general review of glycosylation and secretion of proteins is presented. It is generally recognised that glycosylation, which may be either N-linked, O-linked, or both, is important for efficient transport through the secretory pathway, cf. Caplan et al., Journal of Bacteriology, Vol. 173, No.2, p. 627-635; and Jars et al., The Journal of Biological Chemistry, Vol. 270,

No. 42, p 24810-24817. Moreover, due to the extensive glycosylation the purification of secreted propeptides is difficult and differs considerably from the processing steps that are typically employed for the purification of the mature secreted polypeptide. Clements et al., Gene, 106 (1991) 267-272, have shown that using a eucaryotic consensus signal sequence and two 19-aa pro-sequences comprising fractions of the α-Factor leader and identical except for the presence or absence of a potential Asn linked (N-linked) glycosylation site for secretion of hEGF from yeast had no effect on secretion, and the level of secretion was comparable to the level obtained when using the α-Factor prepro-sequence (about 3μg/ml).

Expression of heterologous proteins as fusion proteins is a well known concept and has been utilized in various contexts in different organisms. Secretory expression of a heterologous protein in yeast is often performed as a fusion protein with a secretion prepro-leader to confer secretion competence. Prepro-leaders tend to be hyperglycosylated or extensively O-linked glycosylated in the S. cerecisiae secretory pathway. Purification of hyperglycosylated fusion protein is laborious due to its heterogeneous nature. Efficient prepro-leaders lacking hyperglycosylation, with no or limited O-linked glycosylation and replacement of the dibasic Kex2 endoprotease site with a more convenient enzymatic processing site, provide an alternative to conventional yeast expression by purification of the fusion protein and subsequently in vitro maturation with a suitable enzyme as exemplified herein for the insulin precursor. In vitro maturation of a purified fusion protein is more flexible since dependency on the Kex2 endoprotease is eliminated and any proteolytic enzyme can be used for maturation provided that the heterologous protein does not have any internal processing sites. Purification of the fusion protein from the culture supernatant followed by in vitro maturation will avoid N-terminal processing of the heterologous protein by dipeptidyl aminopeptidase. Secretion of a fusion protein rather than the heterologous protein has the advantage that the propeptide may increase stability and solubility until purification and maturation. Secretory expression in yeast of heterologous proteins with internal dibasic sites may lead to Kex2 endoprotease processing and a decrease in fermentation yield. This can be avoided by utilizing a secretion prepro-leader lacking N-

linked glycosylation to confer secretion competence, introduction of a suitable enzyme processing site between the prepro-leader and the heterologous protein, expression in a Kex2 endoprotease negative S. cerevisiae strain followed by purification and in vitro maturation.

It is an object of the present invention to provide novel synthetic leader peptides or pro-sequences which ensure a higher yield and a more efficient recovery and/or processing of polypeptides, preferably secreted polypeptides, including leader bound polypeptides, and polypeptides being fused N-terminally to peptide sequences including leader sequences and/or spacer sequences each of which optionally being separated from the other constituent sequences by a processing site, expressed in a eucaryotic host cell organism, preferably a fungal cell, such as a yeast cell or a filamentous fungus cell.

SUMMARY OF THE INVENTION

A novel type of synthetic leader peptide has been found which allows secretion in high yield and/or improved recovery of a polypeptide produced in yeast.

Accordingly, the present invention relates to a DNA construct encoding a polypeptide and having the structure SP-LP-(PS)-(S)-(PS)-*gene*, wherein SP is a DNA sequence (presequence) encoding a signal peptide, LP is a DNA sequence encoding a synthetic leader peptide (propeptide) wherein N-linked glycosylation is lacking, PS is a DNA sequence encoding a protease processing site which is optional, S is a DNA sequence encoding a spacer peptide, and *gene* is a DNA sequence encoding a polypeptide. The structure SP-LP-(PS)-(S)-(PS)-*gene* comprises the following structures, SP-LP-PS-S-PS-*gene*, SP-LP-PS-*gene*, SP-LP-PS-S-*gene*, SP-LP-S-*gene*, SP-LP-S-PS-*gene*, and SP-LP-*gene*; in structures containing more than one PS these may be the same or different.

Preferably, PS is a DNA sequence encoding a yeast protease processing site, such as an endopeptidase processing site, and LS is preferably a DNA sequence encoding a synthetic leader peptide or prepro-leader with the general formula I:

Q/SPIDDTESQTTSVNLMADDTESA/RFATYTXLDVVN/GL(ISMA)/(PGA)KR (I)

wherein

X is a codable amino acid or preferably a sequence of from 1 to 5 codable amino acids which may be the same or different, and is preferably selected from the group consisting of T,L,A,V,D,P,H,N,S,G, and Y is a codable amino acid selected from the group consisting of Q and N; the C-terminal KR is an optional dibasic processing site.

More preferably, LS is a DNA sequence encoding a synthetic leader peptide with the general formula II:

QPIDD(A/D)E(A/D)Q(A/D)(A/D)(A/D)VNLMADD(A/D)E(A/D)AFA(A/D)Q(A/D)PLALDV VNLI(A/D)MAKR (II)

wherein (A/D) can be any codable amino acid, but preferably is alanine (A), serine (S), or aspartic acid (D); the C-terminal KR is an optional dibasic processing site,

or LS is a DNA sequence encoding a synthetic leader peptide with the general formula III:

QPIDD(A/D)E(A/D)Q(A/D)(A/D)(A/D)VNLMADD(A/D)E(A/D)AFA(A/D)Q(A/D)PLALDV VNLI(A/D)MA (III)

wherein (A/D) can be any codable amino acid, but preferably is alanine (A), serine (S), or aspartic acid (D). In formulas I and II above, the C-terminal amino acids KR define a yeast processing site which is optional.

In the present context, the expression "leader peptide" is understood to indicate a pro-peptide sequence whose function is to allow the expressed polypeptide product of *gene* optionally fused at its N-terminal to a spacer peptide and/or a sequence of one or more amino acids defining a processing site, to be directed from the endoplasmic reticulum to the Golgi apparatus and further to a secretory vesicle for secretion into the

medium, (i.e. exportation of the expressed polypeptide across the cellular membrane and cell wall, if present, or at least through the cellular membrane into the periplasmic space of a cell having a cell wall). The term "synthetic" used in connection with leader peptides is intended to indicate that the leader peptide is one not found in nature, and, especially, the leader peptide sequences of the present invention do not include the α-factor leader sequence or fragments and constructs thereof such as the sequence QPVISTTVGSAAEGSLDKR, and a leader sequence derived from S. cerevisiae HSP150 protein having extensive O-linked glycosylation, cf. Simonen, M., Vihinen, H., Jamsa, E., Arumae, U., Kalkkinen, N., and Makarow, M. (1996) The hsp150D-carrier confers secretion competence to the rat nerve growth factor receptor ectodomain in Saccharomyces cerevisiae. Yeast 12, 457-466. Jamsa E ; Holkeri H ; Vihinen H ; Wikstrom M ; Simonen M ; Walse B ; Kalkkinen N ; Paakkola J ; and Makarow M (1995) Structural features of a polypeptide carrier promoting secretion of a beta-lactamase fusion protein in yeast. YEAST 11,1381-91.

The term "signal peptide" is understood to mean a pre-sequence which is predominantly hydrophobic in nature and present as an N-terminal sequence of the precursor form of an extracellular protein, preferably when expressed in yeast. The function of the signal peptide is to allow the expressed protein to be secreted to enter the endoplasmic reticulum. The signal peptide is normally cleaved off in the course of this process. The signal peptide may be heterologous or homologous to the organism producing the protein.

The expression "polypeptide" is intended to indicate a heterologous polypeptide, i.e. a polypeptide or protein which is not produced by the host organism, preferably yeast, in nature as well as a homologous polypeptide, i.e. a polypeptide which is produced by the host organism, preferably a yeast, in nature and any preform thereof. In a preferred embodiment, the DNA construct of the present invention encodes a heterologous polypeptide.

The expression "a codable amino acid" is intended to indicate an amino acid which can be coded for by a triplet ("codon") of nucleotides.

When, in the amino acid sequences given in the present specification, the one or three letter codes of two amino acids, separated by a slash, are given in brackets, e.g. (D/E), this is intended to indicate that the sequence has either the one or the other of these amino acids in the pertinent position.

The expression "heterologous protein" is intended to indicate a protein or polypeptide which is not produced by the host organism in nature, preferably the protein or polypeptide is heterologous in yeast.

The expression "spacer peptide" is intended to indicate an oligopeptide sequence of one or more amino acid residues, preferably 1 to 12 amino acid residues, more preferably about 4 to 6 amino acid residues, such as EEAEPK, EEGEPK, E(EA)3EPK, and EEPK, which may include a processing site, preferably situated N-terminally and/or C-terminally.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is further illustrated with reference to the appended drawings wherein

Fig. 1 shows the expression plasmid pAK773 containing genes expressing the N-terminally extended polypeptides of the invention. In Fig. 1 the following symbols are used: TPI-PROMOTER: Denotes the TPI gene promoter sequence from S. cerevisiae. 2: Denotes the region encoding a signal/leader peptide (e.g. from the YAP3 signal peptide and LA19 leader peptide in conjunction with the EEGEPK N-terminally extended MI3 insulin precursor). TPI-TERMINATOR: Denotes TPI gene terminator sequence of S. cerevisiae. TPI-POMBE: Denotes TPI gene from S. pombe. Origin: Denotes a sequence

from S. cerevisiae 2 μ plasmid including its origin of DNA replication in *S. cerevisiae*. AMP-R: Sequence from pBR322 /pUC13 including the ampicillin resistance gene and an origin of DNA replication in E. coli.

Fig. 2 shows an example of a DNA sequence pAK855 (SEQ ID No. 1) encoding the YAP3 signal peptide, a leader without potential N-linked glycosylation sites, the TA57 leader, and EEGEPK-MI3 insulin precursor complex.

Fig. 3 shows an example of a DNA sequence (SEQ ID No. 2) encoding the YAP3 signal peptide, a leader without potential N-linked glycosylation sites, the leader TA67, and MI3 insulin precursor without N-terminally extension complex.

Fig. 4 shows the expression plasmid pAK855 containing genes expressing the leader sequences of the invention.

Fig. 5 shows in vitro conversion of LA34/IP fusion protein by Achromobacter lyticus lysyl specific protease as a plot of the conversion of LA34/IP fusion protein by Sepharose-bound Achromobacter lyticus lysyl specific protease vs. time. A curve for a first order reaction with (pseudo-)equilibrium is fitted to the data points.

Fig. 6 shows mass spectrometry of in vitro maturation of purified LA34 prepro-leader insulin precursor (MI3) fusion protein by Achromobacter lyticus lysyl specific endoprotease.

DETAILED DISCLOSURE OF THE INVENTION

Preferred leader sequences of the invention are shown in Table 1 below.

Table 1

Strain	leader No.	Leader sequence	SEQ ID No.
yAK744	LA23	QPIDDTESQTTSVNLMADDTESRFATQTTLALDVVNLISMAKR	3
yAK857	TA54	QPIDDTESQTTSVNLMADDTESRFATQTPLALDVVNLISMAKR	4
yAK858	TA56	QPIDDTESQTTSVNLMADDTESRFATNTNALDVVNLISMAKR	5
yAK862	TA57	QPIDDTESQTTSVNLMADDTESAFATQTNSGGLDVVGLISMAKR	6
yAK861	TA59	QPIDDTESQTTSVNLMADDTESAFATQTTSVGGLDVVGLISMAKR	7
	LA64	QPIDDTESQTTSVNLMADDTESRFATQTTLALDVVNLPGAKR	8
	TA65	QPIDDTESQTTSVNLMADDTESAFATQTNSGGLDVVGLPGAKR	9
	TA101	QPIDDTESQTTSVNLMADDTESAFATQTNSGGLDVVGLISMA	10
	TA67	QPIDDTESQTTSVNLMADDTESAFATQTTSVGGLDVVGLPGAKR	11
	TA68	QPIDDTESQTTSVNLMADDTESAFATQTPLALDVVNLISMAKR	12
	LA34	QPIDDTESQTTSVNLMADDTESRFATQTTLALDVVNLISMA	13
	TA76	QPIDDTESQTTSVNLMADDTESRFATQTTLPGAKR	14
	TA77	QPIDDTESQTTSVNLMADDTESRALDVVNLPGAKR	15
	TA78	QPIDDTESQTTSVNLMFATQTTLALDVVNLPGAKR	16
	TA79	QPIDDTESQADDTESRFATQTTLALDVVNLPGAKR	17
	TA80	QPTTSVNLMADDTESRFATQTTLALDVVNLPGAKR	18
	TA89	QPIDDTESQTTSVNLMADDTESAFATQTNSGGLDVVGNTTLISMAKR	19
	TA90	QPIDDTESQTTSVNLMADDTESAFATQTNSGGLDVVGLINTTMAKR	20

In the sequences of Table 1 the C-terminal KR defines a dibasic protease processing site.

Further preferred leader sequences of the invention are shown in Table 1a and 1b below.

Table 1a

Leader No.	Leader Sequence	SEQ ID No.
TA75	QPIDD(A/D)E(A/D)Q(A/D)(A/D)(A/D)VNLMADD(A/D)E(A/D)AFA(A/D)Q(A/D)PLAL DVVNLISMA	21
TA75.50	QPIDDAEAQAAAVNLMADDDEGFAAQAPLALDVVNLISMA	22
TA75.15	QPIDDAEAQDDDVNLMADDDGRFADQAPLALDVVNLISMA	23
TA75.4	QPIDDAEAQDAAVNLMADDGRLKIRFAAQAPLALDVVNLISMA	24

TA75.51	QPIDDAEDQAAAVNLMADDDEDGFAAQAPLALDVVNLISMA	25
TA75.58	QPIDDAEAQDDDVNLMADDDGRFAAQAPLALDVVNLISMA	26
TA75.64	QPIDDAEAQDDDVNLMADDDGRFAAQAPLALDVVNLISMA	27
	and any of the above where SMA is replaced by X^1MA, wherein X^1 may be any codable amino acid, preferably hydrophilic amino acids	28

Table 1b

Leader No.	Leader Sequence	SEQ ID No.
TA91	QPTTSVNLMADDTESAFATQTNSGGLDVVGLISMAKR	29
TA92	QPIDDTESQADDTESAFATQTNSGGLDVVGLISMAKR	30
TA93	QPIDDTESQTTSVNLMFATQTNSGGLDVVGLISMAKR	31
TA94	QPIDDTESQTTSVNLMADDTESAGGLDVVGLISMAKR	32
TA95	QPIDDTESQTTSVNLMADDTESAFATQTNSLISMAKR	33
TA96	QPIDDTESQTTSVNLMADDTESAFATQTNSGGLMAKR	34
TA97	QPIDDTESQTTSVNLMADDTESALISMAKR	35
TA98	QPIDDTESQTTSVNLMLISMAKR	36

The heterologous protein or polypeptide produced by the method of the invention may be any protein which may advantageously be produced in yeast. Preferred examples of such proteins are aprotinin, tissue factor pathway inhibitor or other protease inhibitors, and insulin or insulin precursors, insulin analogues, insulin-like growth factors, such as IGF I and IGF II, human or bovine growth hormone, interleukin, tissue plasminogen activator, glucagon, glucagon-like peptide-1 (GLP 1), glucagon-like peptide-2 (GLP 2), GRPP, Factor VII, Factor VIII, Factor XIII, platelet-derived growth factor, enzymes, such as lipases, or a functional analogue of any one of these proteins. More preferred proteins are precursors of insulin and insulin-like growth factors, and especially the smaller peptides of the proglucagon family, such as glucagon, GLP 1, GLP 2, and GRPP, including truncated forms, such as GLP-1(1-45), GLP-1(1-39), GLP-1(1-38), GLP-1(1-37), GLP-1(1-36), GLP-1(1-35), GLP-1(1-34),

GLP-1(7-45), GLP-1(7-39), GLP-1(7-38), GLP-1(7-37), GLP-1(7-36), GLP-1(7-35), and GLP-1(7-34).

In the present context, the term "functional analogue" is meant to indicate a polypeptide with a similar function as the native protein (this is intended to be understood as relating to the nature rather than the level of biological activity of the native protein). The polypeptide may be structurally similar to the native protein and may be derived from the native protein by addition of one or more amino acids to either or both the C- and N-terminal end of the native protein, substitution of one or more amino acids at one or a number of different sites in the native amino acid sequence, deletion of one or more amino acids at either or both ends of the native protein or at one or several sites in the amino acid sequence, or insertion of one or more amino acids at one or more sites in the native amino acid sequence. Such modifications are well known for several of the proteins mentioned above.

The precursors of insulin, including proinsulin as well as precursors having a truncated and/or modified C-peptide or completely lacking a C-peptide, precursors of insulin analogues, and insulin related peptides, such as insulin-like growth factors, may be of human origin or from other animals and recombinant or semisynthetic sources. The cDNA used for expression of the precursors of insulin, precursors of insulin analogues, or insulin related peptides in the method of the invention include codon optimised forms for expression in yeast.

By "a precursor of insulin" or "a precursor an insulin analogue" is to be understood a single-chain polypeptide which by one or more subsequent chemical and/or enzymatical processes can be converted to a two-chain insulin or insulin analogue molecule having the correct establishment of the three disulphide bridges as found in natural human insulin. Preferred insulin precursors are MI1, B(1-29)-A(1-21); MI3, B(1-29)-Ala-Ala-Lys-A(1-21) (as described in e.g. EP 163 529); X14, B(1-27-Asp-Lys)-Ala-Ala-Lys-A(1-21) (as described in e.g. PCT publication No. 95/00550); B(1-27-Asp-Lys)-A(1-21); B(1-27-Asp-Lys)-Ser-Asp-Asp-Ala-Lys-A(1-21); B(1-29)-Ala-

Ala-Arg-A(1-21) (as described in e.g. PCT Publication No. 95/07931); MI5, B(1-29)-Ser-Asp-Asp-Ala-Lys-A(1-21); and B(1-29)-Ser-Asp-Asp-Ala-Arg-A(1-21), and more preferably MI1, B(1-29)-A(1-21), MI3, B(1-29)-Ala-Ala-Lys-A(1-21) and MI5, B(1-29)-Ser-Asp-Asp-Ala-Lys-A(1-21).

Examples of insulins or insulin analogues which can be produced in this way are human insulin, preferably des(B30) human insulin, porcine insulin; and insulin analogues wherein at least one Lys or Arg is present, preferably insulin analogues wherein Phe^{B1} has been deleted, insulin analogues wherein the A-chain and/or the B-chain have an N-terminal extension and insulin analogues wherein the A-chain and/or the B-chain have a C-terminal extension. Other preferred insulin analogues are such wherein one or more of the amino acid residues, preferably one, two, or three of them, have been substituted by another codable amino acid residue. Thus, in position A21 a parent insulin may instead of Asn have an amino acid residue selected from the group comprising Ala, Gln, Glu, Gly, His, Ile, Leu, Met, Ser, Thr, Trp, Tyr or Val, in particular an amino acid residue selected from the group comprising Gly, Ala, Ser, and Thr. The insulin analogues may also be modified by a combination of the changes outlined above. Likewise, in position B28 a parent insulin may instead of Pro have an amino acid residue selected from the group comprising Asp and Lys, preferably Asp, and in position B29 a parent insulin may instead of Lys have the amino acid Pro. The expression "a codable amino acid residue" as used herein designates an amino acid residue which can be coded for by the genetic code, i. e. a triplet ("codon") of nucleotides.

The signal sequence (SP) may encode any signal peptide which ensures an effective direction of the expressed polypeptide into the secretory pathway of the cell. The signal peptide may be a naturally occurring signal peptide or functional parts thereof or it may be a synthetic peptide. Suitable signal peptides have been found to be the α-factor signal peptide, the signal peptide of mouse salivary amylase, a modified carboxypeptidase signal peptide, the yeast BAR1 signal peptide or the Humicola lanuginosa lipase signal peptide or a derivative thereof. The mouse salivary amylase

signal sequence is described by Hagenbüchle, O. et al., Nature 289 (1981) 643-646. The carboxypeptidase signal sequence is described by Valls, L.A. et al., Cell 48 (1987) 887-897. The BAR1 signal peptide is disclosed in WO 87/02670. The yeast aspartic protease 3 signal peptide is described in Danish patent application No. 0828/93.

The yeast processing site encoded by the DNA sequence PS may suitably be any paired combination of Lys and Arg, such as LysArg, ArgLys, ArgArg or LysLys which permits processing of the polypeptide by the KEX2 protease of Saccharomyces cerevisiae or the equivalent protease in other yeast species (Julius, D.A. et al., Cell 37 (1984) 1075). If KEX2 processing is not convenient, e.g. if it would lead to cleavage of the polypeptide product, e.g. due to the presence of two consecutive basic amino acids internally in the desired product, a processing site for another protease may be selected comprising an amino acid combination which is not found in the polypeptide product, e.g. the processing site for FX_a, IleGluGlyArg (cf. Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1989).

Two of the preferred DNA constructs encoding leader sequences are incorporated in SEQ ID Nos. 1 and 2 as shown in Fig. 2 codon 1078-1209, and Fig. 3 codon 1028-1206, or suitable modifications thereof. Examples of suitable modifications of the DNA sequence are nucleotide substitutions which do not give rise to another amino acid sequence of the protein, but which may correspond to the codon usage of the organism, preferably a fungal organism, such as a yeast, into which the DNA construct is inserted or nucleotide substitutions which do give rise to a different amino acid sequence and therefore, possibly, a different protein structure. Other examples of possible modifications are insertion of one or more codons into the sequence, addition of one or more codons at either end of the sequence and deletion of one or more codons at either end of or within the sequence.

One aspect of the invention is a recombinant expression vector carrying any one of the expression casettes

5'-P-SP-LP-(PS)-(S)-(PS)-*gene*-$(T)_i$-3'

5'-P-SP-LP-PS-*gene*-$(T)_i$-3

5'-P-SP-LP-S-PS-*gene*-$(T)_i$-3'

5'-P-SP-LP-PS-S-*gene*-$(T)_i$-3'

5'-P-SP-LP-S-*gene*-(T)i-3'

5'-P-SP-LP-*gene*-$(T)_i$-3'

5'-P-SP-LP-PS-S-PS-*gene*-$(T)_i$-3'

wherein P is a promoter sequence, SP, LP, PS, S, and *gene*, are as defined above, T is a suitable terminator, e.g. the TPI terminator (cf. Alber, T. and Kawasaki, G., J. Mol. Appl. Genet. 1 (1982) 419-434), and i is 1 or 0. The vector may be any vector which is capable of replicating in yeast organisms. The promoter may be any DNA sequence which shows transcriptional activity in yeast and may be derived from genes encoding proteins either homologous or heterologous to yeast. The promoter is preferably derived from a gene encoding a protein homologous to yeast. Examples of suitable promoters for use in yeast host cells are the Saccharomyces cerevisiae MFα1, TPI, ADH, PGK promoters, or the yeast plasmid 2m replication genes REP 1-3 and origin of replication. The vector may also comprise a selectable marker, e.g. the Schizosaccharomyces pombe TPI gene as described by Russell, P.R., Gene 40 (1985) 125-130.

The expression vector of the invention may be any expression vector that is conveniently subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which the vector is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The methods used to ligate the sequence 5'-P-SP-LS-PS-*gene*-$(T)_i$-3' and to insert it into suitable yeast vectors containing the information necessary for yeast replication, are well known to persons skilled in the art (cf., for instance, Sambrook, J., Fritsch, E.F. and Maniatis, T., op.cit.). It will be understood that the vector may be constructed either by first preparing a DNA construct containing the entire sequence 5'-P-SP-LS-PS-*gene*-$(T)_i$-3' and subsequently inserting this fragment into a suitable expression vector, or by sequentially inserting DNA fragments into a suitable vector containing genetic information for the individual elements (such as the promoter sequence, the signal peptide, the leader sequence GlnProIle(Asp/Glu)(Asp/Glu)X^1(Glu/Asp)X^2 AsnZ(Thr/Ser)X^3, the processing site, the polypeptide, and, if present, the terminator sequence) followed by ligation.

In a further aspect, the present invention relates to a process for producing a polypeptide (or protein) in yeast, the process comprising culturing a yeast cell, which is capable of expressing said polypeptide and which is transformed with a yeast expression vector as described above including a leader peptide sequence of the invention, in a suitable medium to obtain expression and secretion of the said polypeptide, after which the polypeptide is recovered from the medium. The term "culturing" includes fermenting a yeast under laboratory and industrial conditions to produce the polypeptide of interest.

Yeasts are fungi of the class Ascomycetes, subclass Hemiascomycetidae. The yeast organism used in the method of the invention may be any suitable yeast organism which, on cultivation, produces large amounts of the desired polypeptide. Examples of suitable yeast organisms may be strains of the yeast species Saccharomyces cerevisiae, Saccharomyces kluyveri, Saccharomyces uvarum, Schizosaccharomyces pombe, Kluyveromyces lactis, Hansenula polymorpha, Pichia pastoris, Pichia methanolica, Pichia kluyveri, Yarrowia lipolytica, Candida sp., Candida utilis, Candida cacaoi, Geotrichum sp., and Geotrichum fermentans. It is considered obvious for the

skilled person in the art to select any other fungal cell, such as cells of the genus Aspergillus, as the host organism.

The transformation of the yeast cells may for instance be effected by protoplast formation followed by transformation in a manner known per se. The medium used to cultivate the cells may be any conventional medium suitable for growing yeast organisms. The secreted polypeptide, a significant proportion of which will be present in the medium in correctly processed form, may be recovered from the medium by conventional procedures including separating the yeast cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, followed by purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, affinity chromatography or the like.

The invention is further described in the following examples which are not to be construed as limiting the scope of the invention as claimed.

EXAMPLES

Construction of the yeast strain expressing the insulin precursor mediated by leaders lacking N-linked glycosylation.

Synthetic genes coding for the leaders without amino acid sequences potential subjected to attachment of N-linked glycosylation in context with the insulin precursor with or without N-terminal extension of N-terminally extention was constructed using the Polymerase Chain Reaction (PCR). Oligonucleotides for PCR were synthesised using an automatic DNA synthesizer (applied Biosystems model 380A) using phosphoramidite chemistry and commercially available reagents (Beaucage, S.L. and Caruthers, M.H., Tetrahedron letters 22 (1981) 1859-1869). The PCR was performed using the Pwo DNA or EHF Polymerase (Boehringer Mannheim GmbH, Sandhoefer

Strasse 116, Mannheim, Germany) according to the manufacture's instructions and the PCR mix was overlayed with 100 ul mineral oil (sigma Chemical CO, St. Louis MO, USA)

PCR

5 µl oligonucleotide (50 pmol)
5 µl oligonucleotide (50 pmol)
10 µl 10X PCR buffer
8 µl dNTP mix
0.5 µl Pwo or EHF enzyme
0.5 µl pAK680 plasmid as template (0.2 ug DNA)
71 µl dest. water

A total of 12 cycles were performed, one cycle was 94 C for 45 sec.; 40 C for 1 min; 72 C for 1.5 min. The PCR mixture was then loaded onto an 2.5% agarose gel and electrophoresis was performed using standard techniques (Sambrook J, Fritsch EI and Maniatis T, Molecular cloning, Cold Spring Harbour Laboratory press, 1989). The resulting DNA fragment was cut out of the agarose gel and isolated by the Gene Clean kit (Bio 101 inc., PO BOX 2284, La Jolla, CA 92038, USA) according to the manufacturer's instructions.

Certain leader DNA sequences were constructed by overlap PCR reaction as described by Horton, R.M, Cai, Z., Ho, S.N. and Pease, L.R.: Gene splicing by overlap extension: talior-made genes using the polymerase chain reaction. Biotechniques 8 (1990) 528-535.

The purified PCR DNA fragment was dissolved in Des. water and restriction endonucleases buffer and typically cut with the restriction endonucleases BglII and NcoI according to standard techniques (Sambrook J, Fritsch EF and Maniatis T, Molecular cloning, Cold Spring Harbour Laboratory press, 1989). The NcoI-XbaI DNA

fragment on 209 nucleotide basepars was subjected to agarose electrophoresis and purified using The Gene Clean Kit as described.

The expression plasmid pAK721 or a similar plasmid of the cPOT type (see Fig. 1) was typically cut with the restriction endonucleases BglII and XbaI and the vector fragment of 10849 nucleotide basepairs isolated using The Gene Clean Kit as described.

The typically plasmid pAK773 encoding the N-terminally extended EEGEPK-insulin precursor was cut with the restriction endonucleases NcoI and XbaI and the DNA fragment of 209 nucleotide basepars isolated using The Gene Clean Kit as described. The three DNA fragments was ligated together using T4 DNA ligase and standard conditions (Sambrook J, Fritsch EF and Maniatis T, Molecular cloning, Cold spring Harbour laboratory press, 1989). The ligation mix was then transformed into a competent E. coli strain (R-, M+) followed by selection with ampicillin resistance. Plasmid from the resulting E. coli was isolated using standard techniques (Sambrook J, Fritsch EL and Maniatis T, Molecular cloning, Cold spring Harbour laboratory press, 1989), and checked for insert with appropriate restriction endonucleases i.e. BglII, EcoRI, Nco I and XbaI. The selected plasmid was shown by DNA sequence analysis (Sequenase, U.S. Biochemical Corp., USA) to encode the DNA sequence for the leader-MI3 insulin precursor DNA and the DNA encoding the leader to be inserted before the DNA encoding the MI3 insulin precursor DNA.

An example on a DNA sequence pAK855 (SEQ ID No. 1) encoding the YAP3 signal peptide - a leader without potential N-linked glycosylation sites, the TA57 leader, EEGEPK-MI3 insulin precursor complex are shown in Fig. 2.

An example on a DNA sequence (SEQ ID No. 2) encoding the YAP3 signal peptide-synthetic leader without potential N-linked glycosylation sites, the TA69 leader, MI3 insulin precursor without N-terminally extension complex are shown in Fig. 3.

The yeast expression plasmids used are of the C-POT type (see Fig. 1 and 4) and are similar to those described in WO EP 171 142, which contain the Schizosaccharomyces pombe triose phosphate isomerase gene (POT) for plasmid selection and stabilisation in S.cerevisiae. pAK855 also contain the S. cerevisiae triose phosphate isomerase promoter and terminator. The promoter and terminator are similar to those described in the plasmid pKFN1003 (described in WO 90/100075) as are all sequences in plasmid except the sequence between the EcoRI-XbaI fragment encoding the YAP3 signal peptide-leader without N-linked glycosylation-MI3 insulin precursor with or without N-terminally extension.

Purified LA34/IP fusion protein was processed by Sepharose-bound Achromobacter lyticus lysyl specific protease (EC 3.4.21.50) to insulin desB30 (Fig. 5, Fig. 6). From the RP-HPLC analysis results the conversion yield for the removal of the LA34 leader from IP molecule in each collected sample was calculated and then plotted in a graph showing the conversion as a function of the reaction time. A curve for a first-order reaction reaching a (pseudo-)equilibrium can be fitted to the data points as shown in Fig. 5, Fig, 6. Electrospray mass spectrometry was performed on the proteinaceous material isolated from the two main peaks eluted by the RP-HPLC fractionation of the final reaction mixture. For the first eluting peak was found Mw of 5706 Da, corresponding to des(B30)-human insulin (calculated Mw: 5706 Da), and for the second peak was found a Mw of 5625 Da, corresponding to the di-mannosylated LA34-EEAEAEAEPK polypeptide lacking the dipeptide QP (calculated Mw: 5627 Da) the QP dipeptide presumably having been removed by the dipeptidyl aminopeptidase during secretion. This means that within the reaction time an almost complete cleavage of the precursor to an active desB30 insulin molecule has taken place.

CLAIMS

1. A DNA construct encoding a polypeptide and having the structure SP-LP-(PS)-(S)-(PS)-*gene*,

wherein SP is a DNA sequence (presequence) encoding a signal peptide, LP is a DNA sequence encoding a synthetic leader peptide (propeptide) wherein N-linked glycosylation is lacking, PS is a DNA sequence encoding a protease processing site which is optional in both positions, S is a DNA sequence encoding a spacer peptide which is optional, and *gene* is a DNA sequence encoding a polypeptide.

2. A DNA construct according to claim 1, and having the structure SP-LP-PS-*gene*,

wherein SP, LP, PS, and *gene* have the meanings defined above.

3. A DNA construct according to claim 2, which furthermore comprises a sequence encoding a spacer peptide located at the 5' end of *gene* and optionally comprises a sequence encoding a protease processing site located between the 3' end of the sequence encoding said spacer peptide and the 5' end of said *gene*

4. A DNA construct according to any one of the preceding claims which is furthermore characterised in that O-linked glycosylation of LP is lacking.

5. A DNA construct according to any one of claims 1, 2 and 3 which is furthermore characterised in LP having O-linked glycosylation.

6. A DNA construct according to any one of the preceding claims, characterised in that LP does not comprise the consensus N-linked glycosylation sites NXT/S, wherein X designates any codable amino acid.

7. A DNA construct according to any one of the preceding claims, wherein SP is a DNA sequence selected from the group of DNA sequences encoding the S. cerevisiae α-factor signal peptide, the signal peptide of mouse salivary amylase, the yeast carboxypeptidase signal peptide, the yeast aspartic protease 3 signal peptide or the yeast BAR1 signal peptide.

8. A DNA construct according to any one of the preceding claims, wherein LP is a DNA sequence encoding a leader peptide with the general formula I:

Q/SPIDDTESQTTSVNLMADDTESA/RFATYTXLDVVN/GL(ISMA)/(PGA)KR (I)

wherein

X is a codable amino acid or preferably a sequence of from 1 to 5 codable amino acids which may be the same or different, and is preferably selected from the group consisting of T,L,A,V,D,P,H,N,S,G, and **Y** is a codable amino acid selected from the group consisting of Q and N.

9. A DNA construct according to claim 8, wherein **Y** is Q and **X** does not comprise S or T.

10. A DNA construct according to any one of claims 1 to 7, wherein LS is a DNA sequence encoding a synthetic leader peptide with the general formula II:

QPIDD(A/D)E(A/D)Q(A/D)(A/D)(A/D)VNLMADD(A/D)E(A/D)AFA(A/D)Q(A/D)PLALD VVNLI(A/D)MAKR (II)

wherein (A/D) can be any codable amino acid, but preferably is alanine (A) or aspartic acid (D).

11. A DNA construct according to any one of claims 1 to 7, wherein LS is a DNA sequence encoding a synthetic leader peptide with the general formula III:

QPIDD(A/D)E(A/D)Q(A/D)(A/D)(A/D)VNLMADD(A/D)E(A/D)AFA(A/D)Q(A/D)PLAL DVVNLI(A/D)MA (III)

wherein (A/D) can be any codable amino acid, but preferably is alanine (A), serine (S), or aspartic acid (D).

12. A DNA construct according to any one of the preceding claims, wherein X is selected from the sequences NA, TLA, DLA, PLA, TLAGG, TLADGG, TLADD, TLAGD, NSGG, TNSGG, and TSVGG.

13. A DNA construct according to any one of the preceding claims, wherein the leader peptide coded for by the DNA sequence LP is selected from the group comprising the sequences LA23, TA54, TA56, TA57, TA59, LA64, TA65, TA67, TA68, TA76, TA77, TA78, TA79, TA80, TA89, TA90, and TA101 of Table 1 herein.

14. A DNA construct according to any one of the preceding claims, wherein the leader peptide coded for by the DNA sequence LP is selected from the group

comprising the sequences TA75, TA75.50, TA75.15, TA75.4, TA75.51, TA75.58, and TA75.64 of Table 1a herein.

15. A DNA construct according to any one of the preceding claims, wherein the leader peptide coded for by the DNA sequence LP is selected from the group comprising the sequences TA91, TA92, TA93, TA94, TA95, TA96, TA97, and TA98, of Table 1b herein.

16. A DNA construct according to any one of the preceding claims, wherein PS is a DNA sequence encoding an endoprotease processing site which allows in vivo processing.

17. A DNA construct according to the preceding claim wherein the processing site is selected from DNA sequences encoding a dibasic processing site, preferably encoding the amino acid sequences KR, RK, RR, or KK.

18. A DNA construct according to any one of the preceding claims, wherein PS is a DNA sequence encoding an endoprotease processing site which allows in vitro processing.

19. A DNA construct according to the preceding claim wherein the processing site is selected from DNA sequences encoding a monobasic or dibasic processing site, preferably encoding the amino acid sequences K, R, or KR, RK, RR, or KK.

20. A DNA construct according to any one of the preceding claims, wherein the polypeptide is a polypeptide which is heterologous to yeast.

21. A DNA construct according to the preceding claim, wherein the polypeptide is selected from the group consisting of aprotinin, tissue factor pathway inhibitor, or other protease inhibitors, insulin or insulin precursors, insulin-like polypeptides, such as insulin-like growth factor I and insulin-like growth factor II, human or bovine growth hormone, interleukin, glucagon, glucagon-like peptide 1, glucagon-like peptide II, GRPP, tissue plasminogen activator, transforming growth factor a or b, platelet-derived growth factor, enzymes, or a functional analogue thereof.

22. A DNA construct according to claim 18, wherein the polypeptide is selected from the group consisting of insulin or insulin precursors, insulin-like polypeptides, such as insulin-like growth factor I and insulin-like growth factor II, glucagon, glucagon-like peptide 1, glucagon-like peptide II, GRPP, or a functional analogue thereof.

23. A DNA construct according to any one of claims 1 to 17, wherein the polypeptide is a polypeptide which is homologous to yeast.

24. A DNA construct according to the preceding claim, wherein the polypeptide is selected from the group consisting of the gene products of the KEX2 gene, and the YAP3 gene.

25. A DNA construct according to any one of the preceding claims which furthermore comprises a promoter sequence located at the N-terminal end of the structure SP-LP-PS-*gene*.

26. A DNA construct according to any one of the preceding claims which furthermore comprises a promoter sequence located at the N-terminal end of the structure SP-LP-(PS)-(S)-(PS)-*gene*.

27. A DNA construct according to claim 25 and 26, wherein the promoter sequence is a yeast promoter sequence, preferably the TPI promoter.

28. An expression cassette comprising the DNA construct according to claim 25, which additionally comprises a 5' terminally located promoter sequence and a terminator sequence $(T)_i$ located at the 3' terminal of the structure SP-LP-PS-*gene*, where i is 0 or 1.

29. An expression cassette comprising the DNA construct according to claim 26, which additionally comprises a 5' terminally located promoter sequence and a terminator sequence $(T)_i$ located at the 3' terminal of the structure SP-LP-(PS)-(S)-(PS)-*gene*, where i is 0 or 1.

30. An expression cassette according to claims 28 and 29, wherein i is 1 and T is a DNA sequence encoding the TPI terminator.

31. A yeast expression vector comprising the DNA construct according to any of the preceding claims.

32. A yeast cell which is capable of expressing a polypeptide and which is transformed with a yeast expression vector according to claim 31.

33. A yeast cell according to claim 32 selected from the group consisting of Saccharomyces cerevisiae, Saccharomyces uvae, Saccharomyces kluyveri, Schizosaccharomyces pombe, Sacchoromyces uvarum, Kluyveromyces lactis, Hansenula polymorpha, Pichia pastoris, Pichia methanolica, Pichia kluyveri,

Yarrowia lipolytica, Candida sp., Candida utilis, Candida cacaoi, Geotrichum sp., and Geotrichum fermentans.

34. A process for producing a polypeptide in yeast, the process comprising culturing a yeast cell, which is capable of expressing the desired polypeptide and which is transformed with a yeast expression vector according to claim 31, in a suitable medium to obtain expression and secretion of the polypeptide, after which the polypeptide is recovered from the medium.

35. A process according to the preceding claim, wherein the yeast cell is selected from the group consisting of S. cerevisiae, Saccharomyces uvae, Saccharomyces kluyveri, Schizosaccharomyces pombe, Sacchoromyces uvarum, Kluyveromyces lactis, Hansenula polymorpha, Pichia pastoris, Pichia methanolica, Pichia kluyveri, Yarrowia lipolytica, Candida sp., Candida utilis, Candida cacaoi, Geotrichum sp., and Geotrichum fermentans, preferably Saccharomyces cerevisiae.

36. A DNA sequence encoding a synthetic prepro-leader peptide lacking the consensus N-linked glycosylation sites NXT/S, wherein X designates any codable amino acid which is not P.

37. A DNA sequence according to the preceding claim selected from the group consisting of

Q/SPIDDTESQTTSVNLMADDTESA/RFATYTXLDVVN/GL(ISMA)/(PGA)KR (I)

wherein

X is a codable amino acid or preferably a sequence of from 1 to 5 codable amino acids which may be the same or different, and is preferably selected from the group consisting of T,L,A,V,D,P,H,N,S,G, and Y is a codable amino acid selected from the group consisting of Q and N, and wherein the C-terminal KR is an optional processing site.

38. A DNA sequence according to the preceding claim selected from the group consisting of LA23, TA54, TA56, TA57, TA59, LA64, TA65, TA67, TA68, TA76, TA77, TA78, TA79, TA80, TA89, TA90, and TA101 of Table 1 herein.

39. A DNA sequence according to claim 36 selected from the group consisting of

QPIDD(A/D)E(A/D)Q(A/D)(A/D)(A/D)VNLMADD(A/D)E(A/D)AFA(A/D)Q(A/D)PLALD VVNLI(A/D)MAKR (II)

wherein (A/D) can be any codable amino acid, but preferably is alanine (A), serine (S), or aspartic acid (D), and wherein the C-terminal KR is an optional processing site.

40. A DNA sequence according to the preceding claim selected from the group consisting of TA75, TA75.50, TA75.15, TA75.4, TA75.51, TA75.58, and TA75.64 of Table1a herein.

41. A DNA sequence according to claim 36 selected from the group consisting of QPIDD(A/D)E(A/D)Q(A/D)(A/D)(A/D)VNLMADD(A/D)E(A/D)AFA(A/D)Q(A/D)PLAL DVVNLI(A/D)MA (III)

wherein (A/D) can be any codable amino acid, but preferably is alanine (A), serine (S), or aspartic acid (D).

42. A DNA sequence according to the preceding claim selected from the group consisting of TA91, TA92, TA93, TA94, TA95, TA96, TA97, and TA98, of Table 1b herein.

43. A synthetic prepro-leader peptide lacking the consensus N-linked glycosylation sites NXT/S, wherein X designates any codable amino acid which is not P.

44. A synthetic prepro-leader peptide according to the preceding claim selected from the group consisting of

Q/SPIDDTESQTTSVNLMADDTESA/RFATYTXLDVVN/GL(ISMA)/(PGA)KR (I)

wherein

X is a codable amino acid or preferably a sequence of from 1 to 5 codable amino acids which may be the same or different, and is preferably selected from the group consisting of T,L,A,V,D,P,H,N,S,G, and **Y** is a codable amino acid selected from the group consisting of Q and N, and wherein the C-terminal KR is an optional processing site.

45. A synthetic prepro-leader peptide according to the preceding claim selected from the group consisting of LA23, TA54, TA56, TA57, TA59, LA64, TA65, TA67, TA68, TA76, TA77, TA78, TA79, TA80, TA89, TA90, and TA101 of Table 1 herein.

46. A synthetic prepro-leader peptide according to claim 36 selected from the group consisting of

QPIDD(A/D)E(A/D)Q(A/D)(A/D)(A/D)VNLMADD(A/D)E(A/D)AFA(A/D)Q(A/D)PLALD VVNLI(A/D)MAKR (II)

wherein (A/D) can be any codable amino acid, but preferably is alanine (A), serine (S), or aspartic acid (D), and wherein the C-terminal KR is an optional processing site.

47. A a synthetic prepro-leader peptide according to the preceding claim selected from the group consisting of TA75, TA75.50, TA75.15, TA75.4, TA75.51, TA75.58, and TA75.64 of Table1a herein.

48. A synthetic prepro-leader peptide according to claim 36 selected from the group consisting of

QPIDD(A/D)E(A/D)Q(A/D)(A/D)(A/D)VNLMADD(A/D)E(A/D)AFA(A/D)Q(A/D)PLAL DVVNLI(A/D)MA (III)

wherein (A/D) can be any codable amino acid, but preferably is alanine (A), serine (S), or aspartic acid (D).

49. A synthetic prepro-leader peptide according to the preceding claim selected from the group consisting of TA91, TA92, TA93, TA94, TA95, TA96, TA97, and TA98, of Table 1b herein.

50. The use of a first DNA sequence encoding a synthetic prepro-leader lacking N-linked glycosylation sites for secretion of a protein in fungal cells, such as yeast cells.

51. Use according to the preceding claim wherein said prepro-leader additionally lacks O-linked glycosylation sites.

52. Use according to any of claims 36 to 37, wherein said synthetic prepro-leader has an amino acid sequence selected from the group consisting of Q/SPIDDTESQTTSVNLMADDTESA/RFATYTXLDVVN/GL(ISMA)/(PGA)KR (I)

wherein

X is a codable amino acid or preferably a sequence of from 1 to 5 codable amino acids which may be the same or different, and is preferably selected from the group consisting of T,L,A,V,D,P,H,N,S,G, and Y is a codable amino acid selected from the group consisting of Q and N, and wherein the C-terminal KR is an optional processing site.

53. Use according to the preceding claim wherein said prepro-leader is selected from the group consisting of LA23, TA54, TA56, TA57, TA59, LA64, TA65, TA67, TA68, TA76, TA77, TA78, TA79, TA80, TA89, TA90, and TA101 of Table 1 herein.

54. Use according to any of claims 36 to 37, wherein said synthetic prepro-leader has an amino acid sequence selected from the group consisting of QPIDD(A/D)E(A/D)Q(A/D)(A/D)(A/D)VNLMADD(A/D)E(A/D)AFA(A/D)Q(A/D)PLALD VVNLI(A/D)MAKR (II)
wherein (A/D) can be any codable amino acid, but preferably is alanine (A), serine (S), or aspartic acid (D), and wherein the C-terminal KR is an optional processing site.

55. Use according to the preceding claim wherein said prepro-leader is selected from the group consisting of TA75, TA75.50, TA75.15, TA75.4, TA75.51, TA75.58, and TA75.64 of Table1a herein.

56. Use according to the preceding claim wherein said synthetic prepro-leader has an amino acid sequence selected from the group consisting of QPIDD(A/D)E(A/D)Q(A/D)(A/D)(A/D)VNLMADD(A/D)E(A/D)AFA(A/D)Q(A/D)PLAL DVVNLI(A/D)MA (III)
wherein (A/D) can be any codable amino acid, but preferably is alanine (A), serine (S), or aspartic acid (D).

57. Use according to the preceding claim wherein said synthetic prepro-leader has an amino acid sequence selected from the group consisting of TA91, TA92, TA93, TA94, TA95, TA96, TA97, and TA98, of Table 1b herein.

58. Use according to any of claims 36 to 43, wherein said protein is encoded by a second DNA sequence fused at the 5' end to said first DNA sequence encoding said prepro-leader.

59. Use according to the preceding claim wherein a third DNA sequence encoding a spacer peptide optionally having one or more processing sites is inserted in frame between the 3' end of said first DNA sequence encoding said prepro-leader and the 5' end of said second DNA sequence encoding said protein.

60. Use according to the preceding claim wherein the DNA sequence encoding said spacer peptide is selected from DNA sequences encoding an oligopeptide having 1 to 12 amino acid residues, such as EEAEPK, EEGEPK, $E(EA)_3EPK$, EEPK.

61. Use according to any of claims 36 to 46, wherein said protein is a heterologous protein.

62. Use according to the preceding claim wherein said protein is selected from the group consisting of aprotinin, tissue factor pathway inhibitor, or other protease inhibitors, insulin or insulin precursors, insulin-like polypeptides, such as insulin-like growth factor I and insulin-like growth factor II, human or bovine growth hormone, interleukin, glucagon, glucagon-like peptide 1, glucagon-like peptide II, GRPP, tissue plasminogen activator, transforming growth factor a or b, platelet-derived growth factor, enzymes, or a functional analogue thereof.

63. Use according to any of claims 36 to 48 wherein said protein is insulin or an insulin precursor.

64. Use according to any of claims 36 to 46, wherein said protein is a homologous protein, preferably selected from the group consisting of the gene products of the yeast KEX2 and YAP3 genes.

65. Use according to any of claims 36 to 50 wherein said yeast is selected from the group consisting of S. cerevisiae, Saccharomyces uvae, Saccharomyces kluyveri, Schizosaccharomyces pombe, Sacchoromyces uvarum, Kluyveromyces lactis, Hansenula polymorpha, Pichia pastoris, Pichia methanolica, Pichia kluyveri, Yarrowia lipolytica, Candida sp., Candida utilis, Candida cacaoi, Geotrichum sp., and Geotrichum fermentans, preferably Saccharomyces cerevisiae.

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Fig. 1

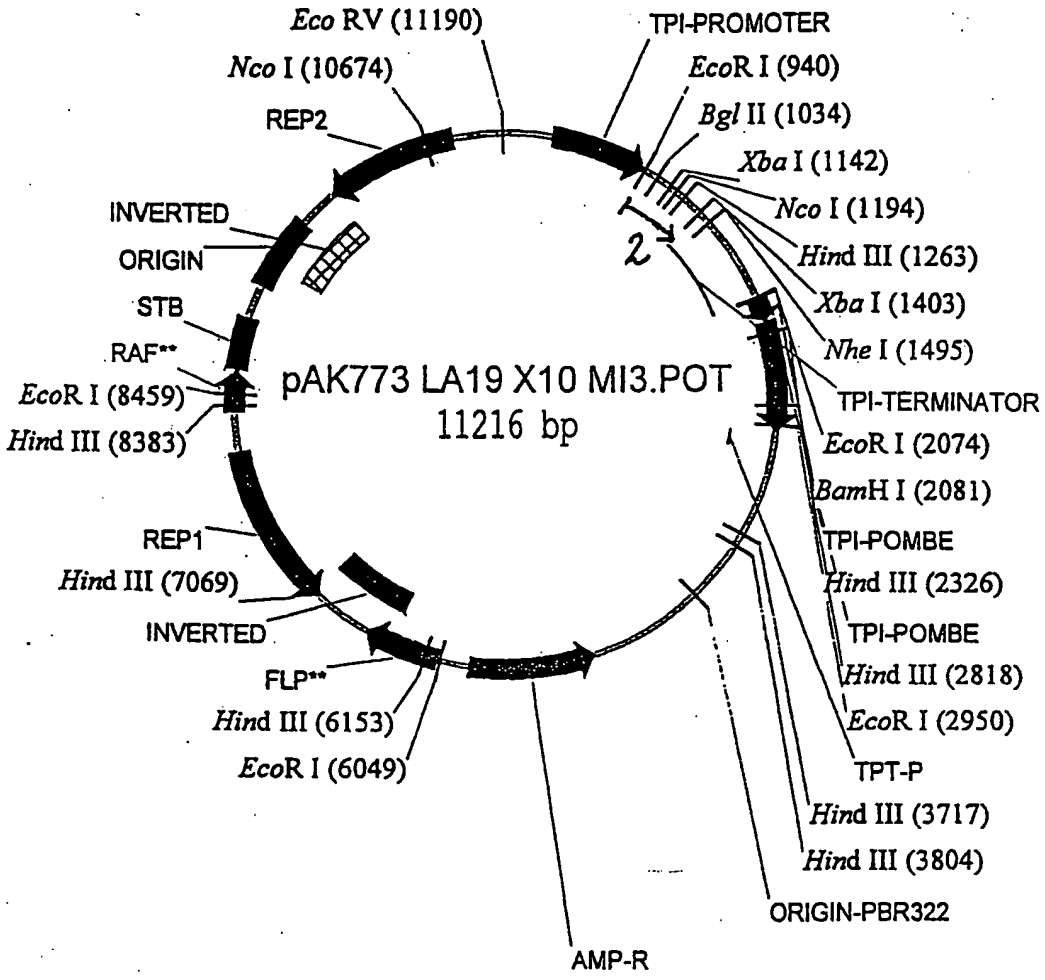


Fig. 2

```
                                                    EcoRI
                                                    ~~~~~~~
 901  TTCTTGCTTA AATCTATAAC TACAAAAAAC ACATACAGGA ATTCCATTCA
      AAGAACGAAT TTAGATATTG ATGTTTTTTG TGTATGTCCT TAAGGTAAGT

 951  AGAATAGTTC AAACAAGAAG ATTACAAACT ATCAATTTCA TACACAATAT
      TCTTATCAAG TTTGTTCTTC TAATGTTTGA TAGTTAAAGT ATGTGTTATA

  +1                  M  K  L  K  T  V  R  S  A  V  L  S
                                        BglII
                                        ~~~~~~
1001  AAACGATTAA AAGAATGAAA CTGAAAACTG TAAGATCTGC GGTCCTTTCG
      TTTGCTAATT TTCTTACTTT GACTTTTGAC ATTCTAGACG CCAGGAAAGC

  +1  S  L  F  A  S  Q  V  L  G  Q  P  I  D  D  T  E  S
                           StyI
                           ~~~~~~
1051  TCACTCTTTG CATCTCAGGT CCTTGGCCAA CCAATTGACG ACACTGAATC
      AGTGAGAAAC GTAGAGTCCA GGAACCGGTT GGTTAACTGC TGTGACTTAG

  +1   Q  T  T  S  V  N  L  M  A  D  D  T  E  S  A  F
1101  TCAAACTACT TCTGTCAACT TGATGGCTGA CGACACTGAA TCTGCTTTCG
      AGTTTGATGA AGACAGTTGA ACTACCGACT GCTGTGACTT AGACGAAAGC

  +1 A  T  Q  T  N  S  G  G  L  D  V  V  G  L  I  S  M
                                                  StyI
                                                  ~~~~~
                                                  NcoI
                                                  ~~~~~
1151  CTACTCAAAC TAACTCTGGT GGTTTGGATG TTGTTGGTTT GATCTCCATG
      GATGAGTTTG ATTGAGACCA CCAAACCTAC AACAACCAAA CTAGAGGTAC

  +1  A  K  R  E  E  G  E  P  K  F  V  N  Q  H  L  C  G
      StyI
      ~
      NcoI
      ~
1201  GCTAAGAGAG AAGAAGGTGA ACCAAAGTTC GTTAACCAAC ACTTGTGCGG
      CGATTCTCTC TTCTTCCACT TGGTTTCAAG CAATTGGTTG TGAACACGCC

  +1   S  H  L  V  E  A  L  Y  L  V  C  G  E  R  G  F
                   HindIII
                   ~~~~~~~
1251  TTCCCACTTG GTTGAAGCTT TGTACTTGGT TTGCGGTGAA AGAGGTTTCT
      AAGGGTGAAC CAACTTCGAA ACATGAACCA AACGCCACTT TCTCCAAAGA

  +1 F  Y  T  P  K  A  A  K  G  I  V  E  Q  C  C  T  S
           Bsu36I
           ~~~~~~~~
1301  TCTACACTCC TAAGGCTGCT AAGGGTATTG TCGAACAATG CTGTACCTCC
      AGATGTGAGG ATTCCGACGA TTCCCATAAC AGCTTGTTAC GACATGGAGG

  +1  I  C  S  L  Y  Q  L  E  N  Y  C  N  *
1351  ATCTGCTCCT TGTACCAATT GGAAAACTAC TGCAACTAGA CGCAGCCCGC
      TAGACGAGGA ACATGGTTAA CCTTTTGATG ACGTTGATCT GCGTCGGGCG

        XbaI
        ~~~~~~
1401  AGGCTCTAGA AACTAAGATT AATATAATTA TATAAAAATA TTATCTTCTT
      TCCGAGATCT TTGATTCTAA TTATATTAAT ATATTTTTAT AATAGAAGAA
```

Fig. 3

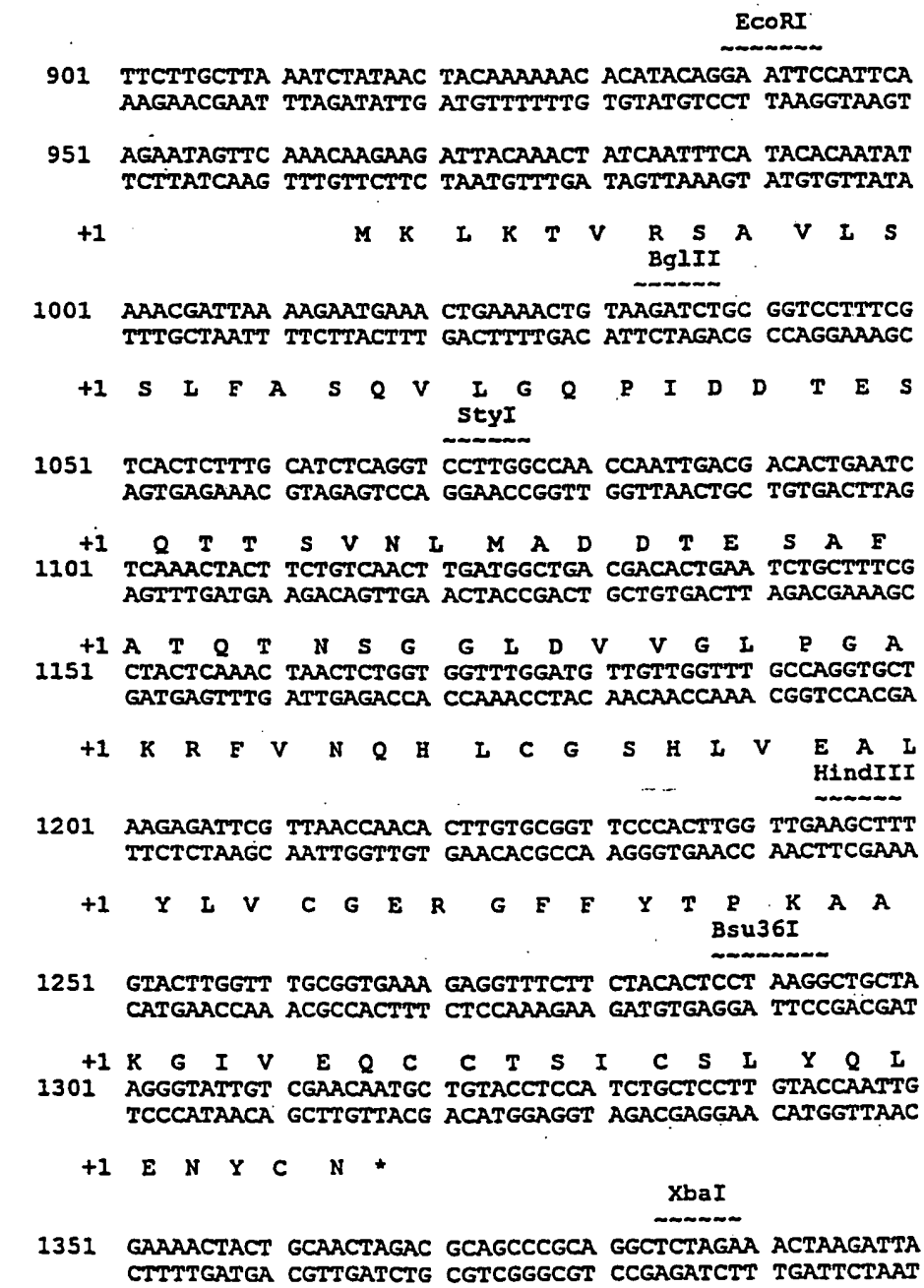

EcoRI
901 TTCTTGCTTA AATCTATAAC TACAAAAAAC ACATACAGGA ATTCCATTCA
AAGAACGAAT TTAGATATTG ATGTTTTTTG TGTATGTCCT TAAGGTAAGT
951 AGAATAGTTC AAACAAGAAG ATTACAAACT ATCAATTTCA TACACAATAT
TCTTATCAAG TTTGTTCTTC TAATGTTTGA TAGTTAAAGT ATGTGTTATA
+1 M K L K T V R S A V L S
BglII
1001 AAACGATTAA AAGAATGAAA CTGAAAACTG TAAGATCTGC GGTCCTTTCG
TTTGCTAATT TTCTTACTTT GACTTTTGAC ATTCTAGACG CCAGGAAAGC
+1 S L F A S Q V L G Q P I D D T E S
StyI
1051 TCACTCTTTG CATCTCAGGT CCTTGGCCAA CCAATTGACG ACACTGAATC
AGTGAGAAAC GTAGAGTCCA GGAACCGGTT GGTTAACTGC TGTGACTTAG
+1 Q T T S V N L M A D D T E S A F
1101 TCAAACTACT TCTGTCAACT TGATGGCTGA CGACACTGAA TCTGCTTTCG
AGTTTGATGA AGACAGTTGA ACTACCGACT GCTGTGACTT AGACGAAAGC
+1 A T Q T N S G G L D V V G L P G A
1151 CTACTCAAAC TAACTCTGGT GGTTTGGATG TTGTTGGTTT GCCAGGTGCT
GATGAGTTTG ATTGAGACCA CCAAACCTAC AACAACCAAA CGGTCCACGA
+1 K R F V N Q H L C G S H L V E A L
HindIII
1201 AAGAGATTCG TTAACCAACA CTTGTGCGGT TCCCACTTGG TTGAAGCTTT
TTCTCTAAGC AATTGGTTGT GAACACGCCA AGGGTGAACC AACTTCGAAA
+1 Y L V C G E R G F F Y T P K A A
Bsu36I
1251 GTACTTGGTT TGCGGTGAAA GAGGTTTCTT CTACACTCCT AAGGCTGCTA
CATGAACCAA ACGCCACTTT CTCCAAAGAA GATGTGAGGA TTCCGACGAT
+1 K G I V E Q C C T S I C S L Y Q L
1301 AGGGTATTGT CGAACAATGC TGTACCTCCA TCTGCTCCTT GTACCAATTG
TCCCATAACA GCTTGTTACG ACATGGAGGT AGACGAGGAA CATGGTTAAC
+1 E N Y C N *
XbaI
1351 GAAAACTACT GCAACTAGAC GCAGCCCGCA GGCTCTAGAA ACTAAGATTA
CTTTTGATGA CGTTGATCTG CGTCGGGCGT CCGAGATCTT TGATTCTAAT

Fig. 4

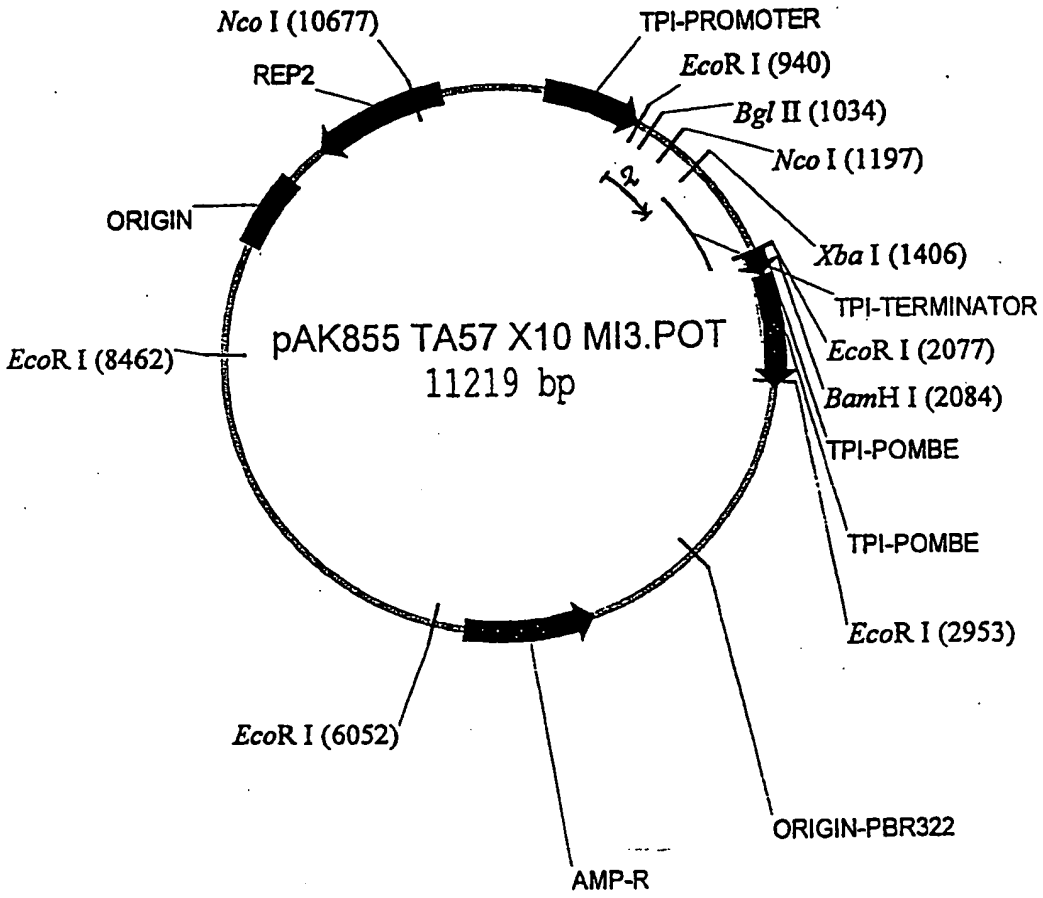

Nco I (10677)
REP2
TPI-PROMOTER
EcoR I (940)
Bgl II (1034)
Nco I (1197)
ORIGIN
Xba I (1406)
TPI-TERMINATOR
pAK855 TA57 X10 MI3.POT
11219 bp
EcoR I (8462)
EcoR I (2077)
BamH I (2084)
TPI-POMBE
TPI-POMBE
EcoR I (2953)
EcoR I (6052)
ORIGIN-PBR322
AMP-R

Fig. 5

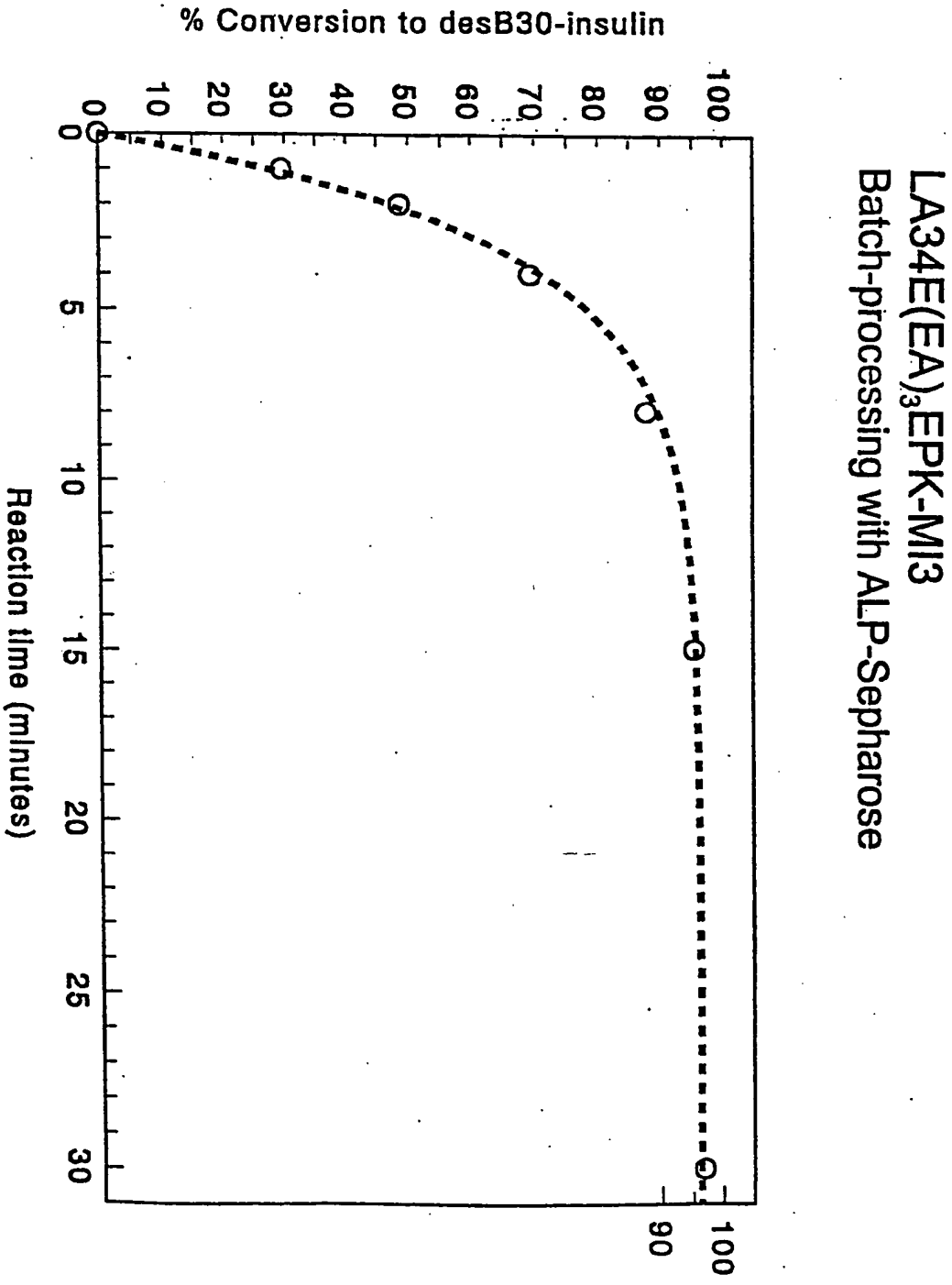
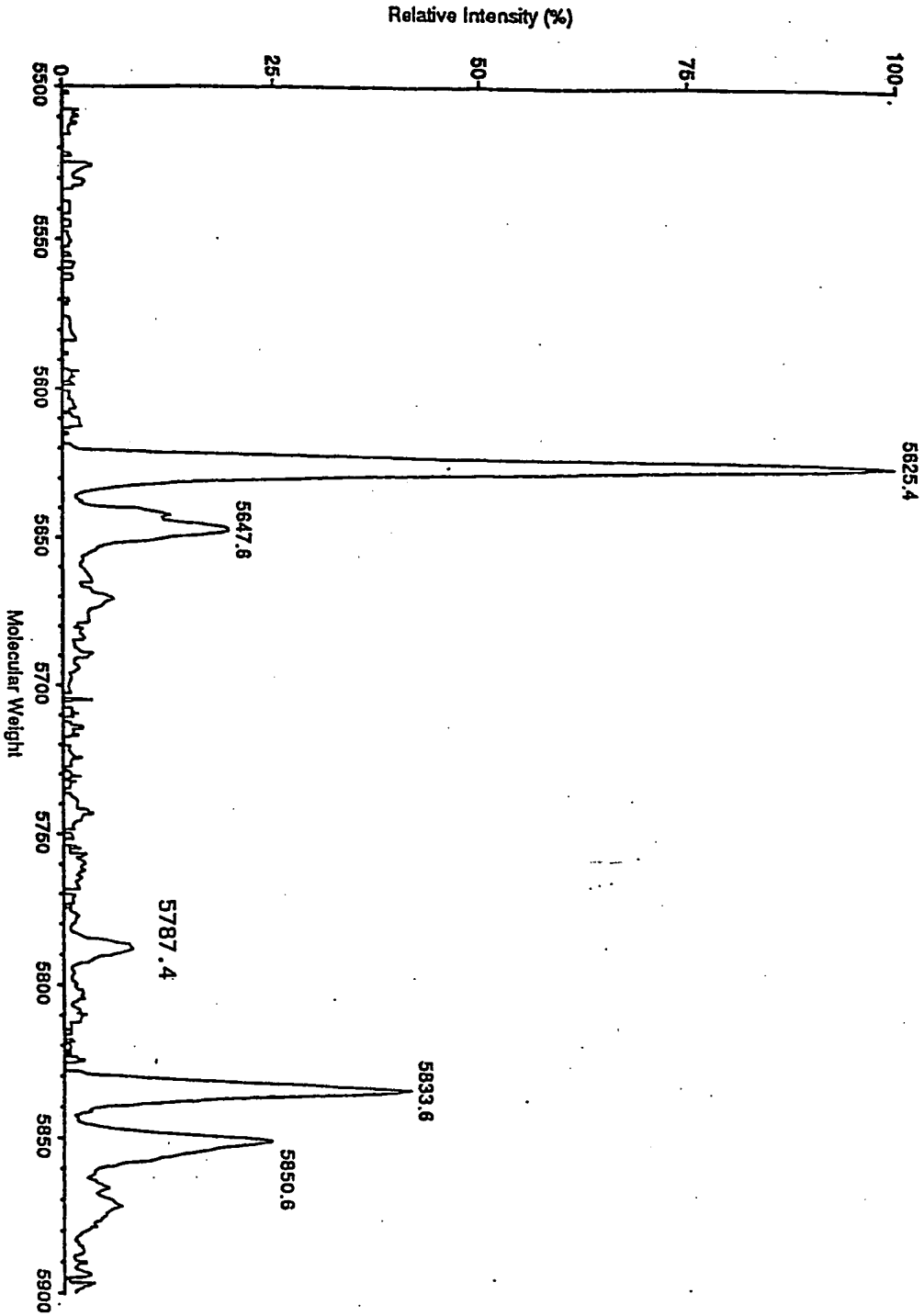

LA34E(EA)3EPK-MI3
Batch-processing with ALP-Sepharose
% Conversion to desB30-insulin
0
10
20
30
40
50
60
70
80
90
100
Reaction time (minutes)
0
5
10
15
20
25
30

Fig. 6


Relative Intensity (%)
0
25
50
75
100
5500
5550
5600
5650
5700
5750
5800
5850
5900
Molecular Weight
5625.4
5647.8
5787.4
5833.6
5850.6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 98/00026

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/81, C07K 14/62
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
D,X	Gene, Volume 106, 1991, John M. Clements et al, "Secretion of human epidermal growth factor from Saccharomyces cerevisiae using synthetic leader sequences" page 267 - page 272	1-7,16-36, 43,50-51, 59-65
D,Y	--	8-15,37-42, 44-49,52-58
X	Abstr. Pap. Am. Chem. Soc. Vol. 196 Meet, MBTD 37, H. M. Sassenfeld et al: "A general strategy for the expression and purification of lymphokines"	1-7,16-36, 43,50-51, 59-65
Y	--	8-15,37-42, 44-49,52

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 July 1998	03 -07- 1998
Name and mailing address of the ISA Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 98/00026

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 9534666 A1 (NOVO NORDISK A/S), 21 December 1995 (21.12.95), the whole document, especially the sequence of claim 21	8-15,37-42, 44-49,52
A		1-7,16-36, 43,50-51, 59-65

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 98/00026

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see nest page

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 98/00026

According to PCT rule 13.2, an international application shall relate to one invention only or a group of inventions linked by one or more of the same or corresponding "special technical features", i.e. features that define a contribution which each of the inventions makes over the prior art. The claimed invention relates to a DNA construct encoding a polypeptide having the structure SP-LP-(PS)-(S)-(PS)-*gene*; the leader peptide (LP) lacks a N-linked glycosylation site. A plausible unifying "special technical feature" could be the LP i.e. a DNA construct having an LP lacking a N-linked glycosylation site, however such an LP is known in the art from, for example, Clements et al., see this search report. No other unifying "special technical feature" has been found. Strictly speaking this application comprises a large number of inventions. However, for the purpose of searching the application has been divided into the following inventions:

Invention 1 (claims 8-12, 14, 37, 39-41, 44, 46-48, 52,54-56 completely and claims 1-7, 13, 15-36, 38, 42-43, 45, 49-51, 53 and 57-65 partially), relates to a DNA construct having any of the general formulas I-III, according to claims 8,10-11 as a leader sequence,

Invention 2 (claims 1-7, 13, 16-36, 38, 43, 45, 50-51, 53 and 58-65 partially), relates to a DNA construct having any of TA76-80 as a leader peptide,

Invention 3 (claims 1-7, 15-36, 42-43, 49-51 and 57-65 partially), relates to a DNA construct having any of TA91,95-98 as a leader peptide.

The search has been restricted to invention 1.

INTERNATIONAL SEARCH REPORT

Information on patent family members

09/06/98

International application No.
PCT/DK 98/00026

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9534666 A1	21/12/95	AU 2733495 A	05/01/96
		CA 2192942 A	21/12/95
		CZ 9603641 A	14/05/97
		EP 0763117 A	19/03/97
		FI 965005 A	13/02/97
		HU 76378 A	28/08/97
		HU 9603477 D	00/00/00
		JP 10501413 T	10/02/98
		NO 965362 A	13/02/97
		PL 317722 A	28/04/97
		US 5639642 A	17/06/97

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